MOLECULAR AND CELLULAR BIOLOGY, Feb. 1992, p. 784–790 Vol. 12, No. 2
0270-7306/92/020784-07$02.00/0

Interaction of *Wnt*-1 Proteins with the Binding Protein BiP

JAN KITAJEWSKI,[1] JOHN O. MASON,[1] AND HAROLD E. VARMUS[1,2]*

Departments of Microbiology and Immunology[1] and Biochemistry and Biophysics,[2] University of California, San Francisco, San Francisco, California 94143-0502

Received 8 August 1991/Accepted 15 November 1991

The mouse *Wnt*-1 gene, a target for insertional activation in mouse mammary tumor virus-induced mammary tumors, encodes poorly secreted, cysteine-rich glycoproteins required for proper central nervous system development. We have been analyzing the biosynthesis of *Wnt*-1 proteins in several cell lines that express *Wnt*-1 cDNA from heterologous promoters. A protein of 78 kDa was found to be associated with the intracellular forms of *Wnt*-1 proteins in mammalian and avian cells by using multiple antisera against *Wnt*-1 proteins. We have identified p78 as the binding protein BiP with anti-BiP antisera and by its release from *Wnt*-1 immunoprecipitates upon incubation with $MgCl_2$ and ATP. Experiments with a *Wnt*-1 mutant that lacks the sequence encoding the signal peptide indicates that *Wnt*-1 proteins must enter the secretory pathway in order to interact with BiP. We demonstrate that *Wnt*-1 proteins are associated with BiP in cells in which active *Wnt*-1 proteins are produced, such as a cultured mammary epithelial cell line and *Wnt*-1 transgenic mouse mammary tumor cells. The association of *Wnt*-1 proteins with BiP may be a factor in determining the efficiency of secretion of *Wnt*-1 gene products.

The *Wnt*-1 gene was originally identified as a target for insertional activation in mouse mammary tumor virus (MMTV)-induced mouse mammary tumors (31, 32, 49), and inappropriate expression of the *Wnt*-1 gene is believed to contribute to transformation of mammary epithelial cells. In support of this proposal, transgenic mice which express the *Wnt*-1 gene in the mammary gland develop mammary hyperplasia and have a high incidence of mammary tumors (47). In addition, expression of a *Wnt*-1 cDNA in cultured mammary epithelial cells leads to morphological transformation and altered growth properties (4, 39). The *Wnt*-1 gene is a member of a large gene family, with at least 10 members identified in the mouse (9, 11, 26, 40, 41, 49). The mouse *Wnt*-1 gene is expressed predominantly in the developing nervous systems of embryos and in spermatids of adult testes (43, 51). Mice homozygous for null mutations in the *Wnt*-1 gene have severe abnormalities of the midbrain and cerebellum, suggesting a critical role for *Wnt*-1 in central nervous system development (24, 46). In *Drosophila melanogaster*, the *Wnt*-1 homolog is the gene wingless, whose protein product is a putative secretory factor that plays a role in establishing segment polarity during embryogenesis (38).

The mouse *Wnt*-1 gene encodes a 370-amino-acid polypeptide that is rich in cysteines, contains four potential N-linked glycosylation sites, and has an amino-terminal hydrophobic signal peptide (9, 49). In cell lines that express *Wnt*-1 cDNA from a heterologous promoter, *Wnt*-1 proteins enter the secretory pathway, where the signal peptide is apparently cleaved (3, 33, 34). Intracellular *Wnt*-1 proteins consist of a 36-kDa unglycosylated form and N-linked glycosylated forms with apparent masses of 38, 40, 42, and 44 kDa. However, *Wnt*-1 proteins are poorly secreted, the majority remaining sequestered intracellularly in endoglycosidase-H-sensitive forms, presumably in the endoplasmic reticulum (ER) and early Golgi stacks prior to the point at which carbohydrate modifications conferring endoglycosidase-H resistance occur. *Wnt*-1 proteins are thus believed to be localized within the ER and early Golgi compartments. In addition, immunofluorescence analysis of *Wnt*-1-expressing cell lines indicated that *Wnt*-1 proteins are localized predominantly within the ER (25, 45). However, endoglycosidase-H-resistant forms of 42 and 44 kDa have been detected in the extracellular medium (33); these forms are believed to be associated with the extracellular matrix or cell surface, since treatment of cultured cells with either suramin (35) or heparin (2) increases the amount in the medium.

The retention of secretory proteins within the ER is often mediated by the immunoglobulin heavy chain-binding protein, BiP (16) (also known as GRP78). The 78-kDa BiP protein is a member of the HSP70 family of heat shock proteins and is found in abundance within the ER (28). BiP binds to various misfolded or unassembled secretory proteins, including aberrantly glycosylated proteins (1, 5, 13, 20, 29), incorrectly disulfide-bonded proteins (20), incompletely assembled multimeric proteins (1, 13, 16, 17), and other misfolded secretory proteins (18). BiP has been proposed to function by stabilizing nascent unfolded proteins until they adopt their correct conformation either by preventing the secretion of unassembled or misfolded proteins or by promoting the folding or assembly of secreted proteins (1, 6, 14, 37, 42). In yeast, BiP function is required for translocation of secretory proteins into the ER (30, 50).

Because *Wnt*-1 proteins are retained within the ER and inefficiently secreted, we examined whether they interact with BiP within the secretory pathway. We report that in several different cell lines expressing *Wnt*-1 proteins the intracellular forms of these proteins are associated with BiP. In addition, *Wnt*-1 proteins must enter the secretory pathway in order to interact with BiP.

MATERIALS AND METHODS

Cell culture. 7dT cells were derived from primary Fisher rat embryo fibroblasts transformed by mutant c-Ha-*ras* (EJ *ras*) and expressing a *Wnt*-1 cDNA under the control of the Moloney murine leukemia virus (Mo-MLV) promoter (3). 7R cells are Fisher rat embryo fibroblasts transformed with mutant c-Ha-*ras*. 7R and 7dT cells were maintained in

* Corresponding author.

Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, dexamethasone (10^{-7} M), and 400 μg of G418 (Geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml. COS-7 cells were maintained in DMEM supplemented with 10% fetal calf serum. Quail QT6 cells (27) were maintained in medium 199 containing Hanks' balanced salt solution supplemented with 2.95 g of tryptose phosphate broth per liter, 5% fetal calf serum, and 1% chicken serum (heat inactivated). C57MG cells (48) were maintained in DMEM supplemented with 10% fetal calf serum and 10 μg of insulin (Sigma Chemical Co.) per ml. C57MG *Wnt*-1 cells are C57MG cells expressing a *Wnt*-1 cDNA under the control of the Mo-MLV promoter and are maintained as C57MG cells with the addition of 400 μg of G418 per ml.

Antisera. Ascites fluid containing mouse monoclonal antibody raised against *Wnt*-1-specific peptide A (residues 200 to 212) and rabbit polyclonal serum against *Wnt*-1-specific peptide B (residues 275 to 289) have been described previously (3). Rabbit serum C34 was generated using *Escherichia coli*-expressed *Wnt*-1 protein as the antigen. Rat anti-mouse BiP monoclonal antibody (1) was kindly provided by David Bole (University of Michigan, Ann Arbor).

Expression in COS and QT6 cells. The generation of a *Wnt*-1 cDNA that no longer encodes the N-terminal hydrophobic leader sequence from codons 2 to 27 has been described previously (23). *Wnt*-1 and *Wnt*-1(ΔL) cDNAs were subcloned into pON expression vectors (12) that utilize the cytomegalovirus immediate early promoter/enhancer to drive expression. The plasmids pON *Wnt*-1 and pON *Wnt*-1(ΔL) were generated by inserting 1.9-kb *Xba*I-*Hin*dIII fragments (the *Xba*I staggered end was made flush by using Klenow fragment) bearing *Wnt*-1 cDNAs into *Pvu*II-*Hin*dIII-digested plasmid pON 249 (12).

COS-7 cells were transfected with 5 μg of plasmid per 100-mm dish in the presence of 400 μg of DEAE-dextran per ml followed by treatment with 10% dimethylsulfoxide in phosphate-buffered saline (PBS) (22). For QT6 cells, calcium phosphate-mediated transfections were performed (15) with 20 μg of plasmid followed by treatment with 10% dimethylsulfoxide in PBS. Cells were analyzed 40 h after transfection.

Immunoprecipitation and ATP-dependent release assay. Subconfluent 100-mm dishes of cells were washed with PBS, preincubated for 15 min at 37°C in DMEM lacking cysteine and methionine (labeling medium), and then incubated for 4 h at 37°C with 2.0 ml of labeling medium supplemented with 0.125 mCi of ^{35}S-*trans*-label per ml and 0.125 mCi of [^{35}S] cysteine per ml (ICN Radiochemicals, Irvine, Calif.). After being labeled, cells were washed twice with cold PBS, 1 ml of TENT buffer (1% Triton X-100, 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 2 μg of leupeptin per ml, 1 μg of pepstatin per ml) was added to each dish, and the dishes were rocked for 20 min at 4°C. The lysates were centrifuged for 10 min at 10,000 × *g*, and the supernatants were normalized for protein content. The cell lysates were incubated for 2 to 4 h at 4°C with 2 μl of ascites fluid containing monoclonal antibodies against *Wnt*-1-specific peptide A, 5 μl of rabbit serum C34, or 5 μl of preimmune rabbit serum. Protein A-Sepharose (Sigma Chemical Co.) was used to collect the antigen-antibody complexes. Immunoprecipitates were washed four times with cold TENT buffer and then electrophoresed on 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels.

For the ATP-dependent release assay, immunoprecipitations were carried out on 7R and 7dT cell lysates as described above. After the last wash, the immunoprecipitates were suspended in buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$) in the presence or absence of 100 μM ATP. After 10 min of incubation at 25°C, the Sepharose was pelleted by centrifugation, and the bound and released fractions were analyzed by 12.5% SDS–polyacrylamide gel electrophoresis.

Immunoblot analysis. *Wnt*-1 immunoprecipitates were prepared as described above, fractionated by 10% SDS-polyacrylamide gel electrophoresis, and then electroblotted onto a nitrocellulose filter. The protein blot was blocked for 1 h at 25°C in Tris-buffered saline (TBS) (10 mM Tris, pH 8.0, 150 mM NaCl) containing 2% bovine serum albumin (blocking buffer A), washed once for 5 min with TBST (TBS with 0.05% Tween-20), and then incubated for 4 h at 25°C with rat anti-BiP monoclonal antibodies diluted 1:25 in TBST. The primary antibody was removed, and the blot was washed three time for 5 min each in TBST, incubated for 1 h at 25°C with 1:7,500-diluted alkaline phosphatase-conjugated anti-rat immunoglobulin G (IgG) (Promega) in TBST, and then washed three times in TBST. Enzymatic color development was done for 15 min in buffer containing 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 330 μg of Nitro Blue Tetrazolium per ml, and 165 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml.

For the immunoblot analysis of *Wnt*-1 proteins and BiP in cultured mammary cells (see Fig. 5), immunoprecipitates were made from detergent lysates of subconfluent 100-mm dishes of C57MG cells and C57MG cells expressing a *Wnt*-1 cDNA as described above. For the immunoblot analysis of *Wnt*-1 proteins and BiP in a mammary tumor (see Fig. 5), a mammary tumor from the *Wnt*-1 transgenic line 303 (47) was disrupted by Polytron homogenization in 4 ml of TENT buffer supplemented with 20 μg of leupeptin, 20 μg of pepstatin, 100 μg of aprotinin, and 10 μg of phenylmethylsulfonyl fluoride per ml. The lysate was centrifuged for 30 min at 10,000 × *g*, and the supernatant was precleared with protein A-Sepharose; and immunoprecipitations were then carried out as described above. The immunoprecipitates from cultured mammary cells and a mammary tumor were fractionated on 10% SDS–polyacrylamide gels and then electroblotted onto nitrocellulose filters. One protein blot was blocked for 1 h at 25°C in TBS containing 1% bovine serum albumin and 0.2% Tween-20 (blocking buffer B) and then probed for BiP by using rat anti-BiP monoclonal antibodies followed by alkaline phosphatase-conjugated anti-rat IgG as described above. A separate protein blot, bearing identical samples as the BiP blot, was blocked with blocking buffer B and then incubated overnight at 4°C with monoclonal antibodies against *Wnt*-1-specific peptide A diluted 1:2,000 in blocking buffer B. The primary antibody was removed, and the blot was washed three times for 5 min each in TBS containing 0.2% Tween-20, incubated for 1 h at 4°C with horseradish peroxidase-conjugated anti-mouse IgG antibodies (Amersham) diluted 1:20,000 in blocking buffer B, and then washed three times as described above. Bound antibodies were detected by using the Enhanced Chemiluminescence (ECL) detection system (Amersham).

RESULTS

Intracellular *Wnt*-1 protein coimmunoprecipitate with a 78-kDa protein. The production and processing of *Wnt*-1 proteins were examined in the rat embryo fibroblast cell line 7dT, which expresses *Wnt*-1 cDNA under the control of the

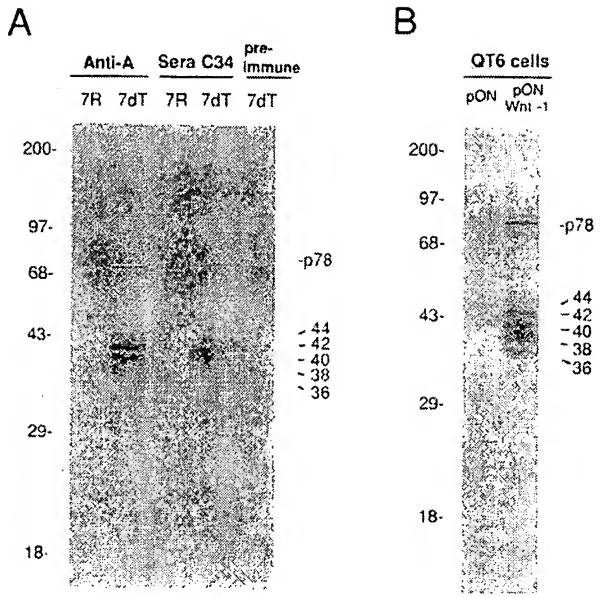

FIG. 1. A 78-kDa protein coimmunoprecipitates with *Wnt*-1 proteins. (A) Different antibodies against *Wnt*-1 proteins coimmunoprecipitate p78. 7R and 7dT cells were metabolically labeled with [^{35}S] cysteine and [^{35}S]methionine; this was followed by detergent lysis. Cell extracts were immunoprecipitated with either anti-*Wnt*-1 peptide A antibodies (anti-A), rabbit serum C34 against *Wnt*-1 proteins (sera C34), or preimmune rabbit serum (preimmune). The immunoprecipitates were washed in buffer containing 1% Triton X-100 and then analyzed by 12.5% SDS–polyacrylamide gel electrophoresis. The positions of the major (40- and 42-kDa) and minor (36, 38, and 44 kDa) *Wnt*-1-specific bands are indicated, as is that of the coimmunoprecipitating band (78 kDa). (B) p78 associates with *Wnt*-1 proteins in transfected QT6 cells. QT6 cells were transfected with either a control vector (pON) or with a vector encoding *Wnt*-1 protein (pON-*Wnt*-1). At 40 h posttransfection, immunoprecipitates with anti-*Wnt*-1 peptide A antibodies were prepared and analyzed as described for Fig. 1A. Numbers to the left of the gels indicate molecular mass (in kilodaltons).

Mo-MLV promoter and is transformed by mutant c-Ha-*ras* (see Materials and Methods). *Wnt*-1 proteins produced in 7dT cells were analyzed by metabolic labeling of cells followed by detergent lysis, and *Wnt*-1 proteins were immunoprecipitated by using ascites fluid containing mouse monoclonal antibody against *Wnt*-1-specific peptide A (see Materials and Methods). As in earlier reports, five forms of *Wnt*-1 proteins were detected: two major species of 40 and 42 kDa (p40 and p42) and minor species of 36, 38, and 44 kDa (p36, p38, and p44) (Fig. 1A). These proteins were not observed in control 7R cells (rat embryo fibroblasts transformed by mutant c-Ha-*ras*). Previous analysis of *Wnt*-1 proteins produced in 7dT cells indicated that p36 is an unglycosylated species that has probably undergone cleavage of the N-terminal signal peptide, whereas p38, p40, p42, and p44 were N-linked-glycosylated derivatives of p36 (3, 34).

We noted that a protein of approximately 78 kDa (p78) coimmunoprecipitated with *Wnt*-1 proteins from 7dT cell extracts but was not detected in 7R cell extracts (Fig. 1A). To demonstrate that p78 was associated with *Wnt*-1 proteins rather than binding nonspecifically to the monoclonal antibody, we repeated the immunoprecipitations from 7dT cells with a rabbit antiserum raised against *Wnt*-1 protein expressed in *E. coli* (serum C34). p78 again coimmunoprecipitated with *Wnt*-1 proteins (Fig. 1A), and neither *Wnt*-1 proteins nor p78 were detected in control 7R cells. Association of p78 with *Wnt*-1 proteins was also observed after immunoprecipitations with a rabbit antiserum directed against a *Wnt*-1-specific peptide B (data not shown). In addition, preimmune rabbit serum failed to immunoprecipitate *Wnt*-1 proteins or p78. Therefore, several different antisera against multiple epitopes of the *Wnt*-1 protein coprecipitated p78 with *Wnt*-1 proteins, implying that p78 associates with one or more forms of *Wnt*-1 proteins in a complex that survives the conditions used for immunoprecipitation. The *Wnt*-1/p78 complexes were very stable, resisting disruption by buffer containing 1% Triton X-100 and 0.1% SDS or by a high salt concentration (data not shown).

The association of p78 with *Wnt*-1 proteins was also seen in other cell lines expressing *Wnt*-1 proteins. Figure 1B shows an analysis of proteins produced in the quail cell line QT6 transiently transfected with expression vectors (pON) expressing *Wnt*-1 proteins (see Materials and Methods). Immunoprecipitations from pON-*Wnt*-1-transfected cells show a pattern of *Wnt*-1 proteins similar to that seen in 7dT cells (Fig. 1A and B). p78 was found to coimmunoprecipitate with *Wnt*-1 proteins and is not present in immunoprecipitates from QT6 cells transfected with a control plasmid.

p78 is the binding protein BiP. Given that the intracellular forms of *Wnt*-1 appear to be sequestered within the ER, the association of p78 was reminiscent of the association of the binding protein BiP with a variety of misfolded or unassembled secretory proteins within the ER (14, 37, 42). BiP is a 78-kDa protein found in the ER (28); therefore, the size and presumed subcellular location of *Wnt*-1-associated p78 were consistent with this proposal.

We investigated whether p78 might be BiP by using immunological criteria. *Wnt*-1 immunoprecipitates from 7R cells, 7dT cells, and transfected QT6 cells were prepared by using several antisera against *Wnt*-1 proteins (as described above). The proteins were fractionated in SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with a rat monoclonal antibody against mouse BiP. p78 was recognized by the anti-BiP antibody in immunoprecipitates from 7dT cells and from QT6 cells expressing *Wnt*-1 proteins but not in immunoprecipitates from 7R cells or control QT6 cells (Fig. 2). Once again, BiP was identified only in immune complexes bearing *Wnt*-1 proteins.

ATP-dependent release of BiP from *Wnt*-1. The BiP protein, like other members of the HSP70 protein family, has an ATPase domain, and ATP has been shown to cause the release of BiP from associated proteins or peptides (6, 28). We therefore tested whether ATP could stimulate the dissociation of BiP from *Wnt*-1 proteins in immunoprecipitates prepared from labeled 7dT cells as described above. The majority of BiP was released from the immune complex after incubation with ATP, whereas in the absence of ATP most BiP remained associated with *Wnt*-1 proteins (Fig. 3). The small amount of BiP released in the absence of ATP may be due to endogenous ATP in the immune complex or to the increase in temperature to 25°C, since previous reports indicate that BiP binding may be sensitive to temperature (18).

***Wnt*-1 proteins must enter the secretory pathway to interact with BiP.** We sought to determine whether entry into the secretory pathway was a prerequisite for association of *Wnt*-1 with BiP. To address this point, we used a mutant cDNA that no longer encodes the N-terminal hydrophobic leader sequence, *Wnt*-1(ΔL). The normal and the leaderless

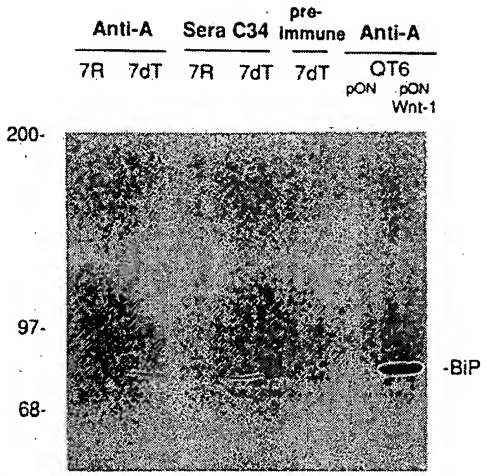

FIG. 2. Antibodies against BiP recognize p78. Immunoprecipitates from 7R cells, 7dT cells, and transfected QT6 cells were prepared as described for Fig. 1 by using either anti-*Wnt*-1 peptide A antibodies (Anti-A), rabbit serum C34, or preimmune rabbit serum as indicated, and then were fractionated on a 10% SDS–polyacrylamide gel. The proteins were transferred to a nitrocellulose filter which was then probed with anti-BiP monoclonal antibodies. Bound antibodies were detected with alkaline phosphatase-conjugated anti-rat IgG as described in Materials and Methods. Numbers on the left indicate molecular mass (in kilodaltons).

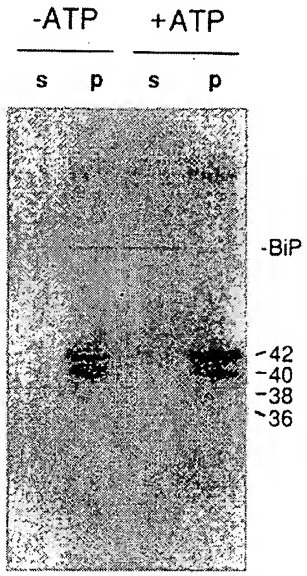

FIG. 3. ATP-dependent release of BiP from *Wnt*-1 immunoprecipitates. *Wnt*-1-containing immunoprecipitates from labeled 7dT cell extracts were prepared with anti-*Wnt*-1 peptide A antibodies as described for Fig. 1. The immunoprecipitates were then resuspended either in buffer alone (−ATP) or in buffer with 100 μM ATP (+ATP) and incubated for 10 min at 25°C. The immune complex was then repelleted, and released material in the supernatant (s) and the pellet (p) was analyzed by 12.5% SDS–polyacrylamide gel electrophoresis. Numbers on the left indicate molecular mass (in kilodaltons).

Wnt-1 cDNAs were subcloned into the pON (12) expression vector and used to transfect COS-7 cells transiently. The pattern of *Wnt*-1 proteins produced in wild-type vector-transfected COS-7 cells (Fig. 4) was similar to that seen in 7dT cells and QT6 cells, and a protein of approximately 78 kDa, which we presume to be BiP, coimmunoprecipitated with *Wnt*-1 proteins.

Immunoprecipitates from *Wnt*-1(ΔL)-expressing COS-7 cells contain a protein of 36 kDa which comigrates with the leader-cleaved, unglycosylated protein in cells transfected with wild-type *Wnt*-1, but the lower-mobility forms were not observed (Fig. 4). Thus, the *Wnt*-1(ΔL) protein, p36, did not appear to be glycosylated, implying that, as expected, leaderless *Wnt*-1 proteins do not enter the secretory pathway. Moreover, the p78 (BiP) protein was not detected in *Wnt*-1 (ΔL) immune complexes. This observation is consistent with the behavior of BiP as an ER luminal protein that interacts with proteins within the secretory pathway. The lack of association of *Wnt*-1(ΔL) with p78 (BiP) also diminishes the possibility that wild-type *Wnt*-1 proteins and BiP become associated after detergent lysis of cells. We conclude that *Wnt*-1 proteins must enter the secretory pathway in order to associate with BiP.

***Wnt*-1 proteins associate with BiP in mammary cells and mammary tumors.** The experiments described thus far have been performed with fibroblasts programmed to produce *Wnt*-1 proteins. Such cells are able to generate biologically active *Wnt*-1 gene products, since they can serve as donors in paracrine assays (19, 36). Nevertheless, fibroblasts have not been shown to express endogenous *Wnt*-1 genes, so it could be argued that interactions between BiP and *Wnt*-1 proteins do not represent a naturally occurring phenomenon. For this reason, we have performed additional experiments with cultured mammary cells and primary mammary carcinomas, in which *Wnt* genes are known to have biologically significant roles. For example, MMTV-induced mammary cancer is associated with insertion mutation of *Wnt*-1 and *Wnt*-3 (31, 32, 41, 49); an MMTV-*Wnt*-1 transgene causes hyperplasia and neoplasia of mammary glands (47); expression of *Wnt*-1 in an established mouse mammary epithelial line, C57MG, alters cell growth and morphology (4); and several members of the *Wnt* gene family, albeit not *Wnt*-1, are expressed during mammary gland development (10).

Wnt-1 immunoprecipitates from the C57MG mammary epithelial cell line and a C57MG cell line which expresses *Wnt*-1 cDNA under the control of the Mo-MLV promoter were prepared by using antibodies against *Wnt*-1-specific peptide A (as described above). We also prepared *Wnt*-1 immunoprecipitates from a *Wnt*-1 transgenic mouse mammary tumor by using two sources of antibodies; this transgenic mouse line bears a *Wnt*-1 transgene with the MMTV long terminal repeat positioned 5′ of the first *Wnt*-1 exon in a transcriptional orientation opposite that of the gene. Immunoblots were carried out to ascertain whether *Wnt*-1 proteins (Fig. 5A) and BiP (Fig. 5B) were present in the immunoprecipitates. Two major species of *Wnt*-1 proteins, p40 and p42, were detected in C57MG cells expressing *Wnt*-1 cDNA (Fig. 5A, lane 2) but not in control C57MG cells (Fig. 5A, lane 1). The p40 and p42 *Wnt*-1 species were also detected in a *Wnt*-1 transgenic mouse mammary tumor when *Wnt*-1-specific antibodies were used in immunoprecipitations (Fig. 5A, lanes 3 and 5), but not when preimmune rabbit serum was used (Fig. 5A, lane 4). BiP was detected in immunoprecipitates in which *Wnt*-1 proteins were present (Fig. 5B, lanes 2, 3, and 5) but not in control immunoprecipitates (Fig. 5B, lanes 1 and 4). We conclude that BiP is

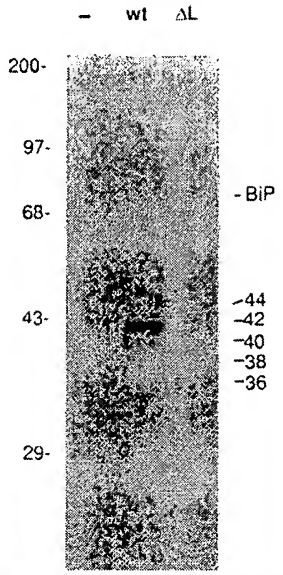

FIG. 4. Leaderless *Wnt*-1 proteins do not associate with BiP. COS-7 cells were mock transfected (−) or transfected with vectors which express either wild-type *Wnt*-1 proteins (wt) or leaderless *Wnt*-1 proteins (ΔL) encoded by mutant *Wnt*-1 cDNA lacking codons 2 through 27. At 40 h posttransfection, cells were metabolically labeled and cell extracts were prepared by detergent lysis as described for Fig. 1. Labeled proteins were immunoprecipitated from the extracts with anti-*Wnt*-1 peptide A antibodies and analyzed by 12.5% SDS–polyacrylamide gel electrophoresis. Numbers indicate molecular mass (in kilodaltons).

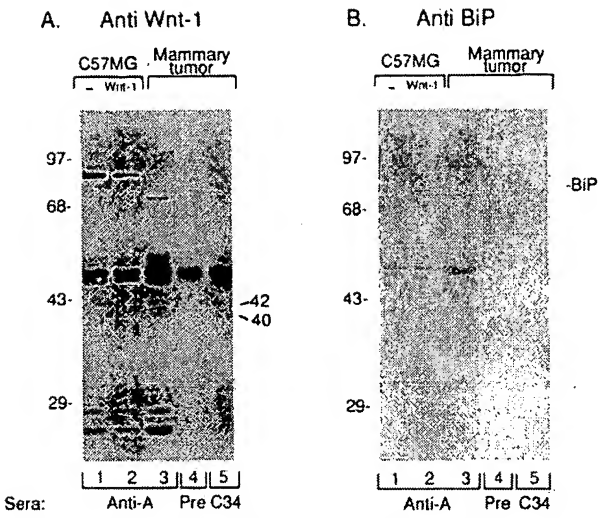

FIG. 5. *Wnt*-1 proteins associate with BiP in mammary cells and mammary tumors. Cell extracts from C57MG cells or C57MG cells expressing *Wnt*-1 proteins were immunoprecipitated with anti-*Wnt*-1 peptide A antibodies (Anti-A). Extracts from a mammary gland tumor of a *Wnt*-1 transgenic mouse (mammary tumor) were immunoprecipitated with either anti-*Wnt*-1 peptide A antibodies (Anti-A), preimmune rabbit sera (Pre), or rabbit serum C34 (C34). The immunoprecipitates were fractionated on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose filter. (A) The filter was probed with anti-*Wnt*-1 peptide A monoclonal antibodies, and bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG as described in Materials and Methods. (B) The filter was probed with anti-BiP monoclonal antibodies, and bound antibodies were detected with alkaline phosphatase-conjugated anti-rat IgG as described in Materials and Methods. −, mock-infected cells. Numbers indicate molecular mass (in kilodaltons).

associated with *Wnt*-1 proteins which are produced in cultured mammary epithelial cells or in mouse mammary gland tumors.

DISCUSSION

We have shown that a 78-kDa protein (p78) is associated with the intracellular forms of *Wnt*-1 proteins and have demonstrated that p78 is the binding protein BiP by immunological, genetic, and biochemical criteria. *Wnt*-1-associated p78 is recognized by rat anti-BiP antibodies, can be released from *Wnt*-1 proteins in an ATP-dependent fashion, and does not associate with a mutant *Wnt*-1 protein that cannot enter the secretory pathway.

Wnt-1 proteins are believed to function as secreted growth factors or morphogens, and BiP is an ER lumenal protein which binds misfolded proteins, preventing their secretion (14, 37). A variety of cell lines expressing *Wnt*-1 cDNA have been used to demonstrate that *Wnt*-1 proteins enter the secretory pathway and are glycosylated but do not transit efficiently through the secretory pathway (2, 25, 33–35, 45). On the basis of the proposed functions of BiP (14, 37), we believe that the majority of the intracellular forms of *Wnt*-1 proteins have not achieved their correct conformation and are retained in the ER through association with BiP. By analyzing the stoichiometry of this interaction, we found that the majority of *Wnt*-1 proteins are bound to BiP (21), suggesting that BiP binding is the primary determinant for the retention of *Wnt*-1 proteins within the ER. A small proportion of *Wnt*-1 proteins produced in these cell lines must achieve their correct conformation, since extracellular *Wnt*-1 proteins have been detected (2, 21, 33, 35).

Mammary epithelial cells expressing *Wnt*-1 proteins undergo morphological transformation and altered growth properties (4, 39), and normal mammary cells express other members of the *Wnt* gene family encoding proteins likely to be structurally similar to *Wnt*-1 proteins (10). We have demonstrated that *Wnt*-1 proteins are associated with BiP in cultured mammary epithelial cells transformed by an exogenous *Wnt*-1 gene and in a *Wnt*-1 transgenic mouse mammary tumor. In these settings, *Wnt*-1 proteins must have achieved their correct conformation, but a proportion of *Wnt*-1 proteins are still bound to BiP and are presumably retained within the ER.

The examination of a mouse mammary tumor provides the first demonstration that *Wnt*-1 proteins are produced in the *Wnt*-1 transgenic mouse line. The processing of *Wnt*-1 proteins appears to be very similar in mammary tumors and in cultured mammary epithelial cells. In addition, we have shown that *Wnt*-1 proteins are associated with BiP in a tissue sample taken from a whole animal.

The determinants of a newly made or misfolded secretory protein that are recognized by BiP are not well defined. BiP will bind to short peptides (6) and exhibits a preference for aliphatic residues (7). BiP has been proposed to interact with exposed hydrophobic patches (37), distinct conformations such as an extended chain, or reduced cysteines that have not been incorporated into disulfide bonds (44). Incomplete glycosylation of several secretory peptides has also been correlated with increased BiP binding (5). The *Wnt*-1 gene

encodes four potential sites of N-linked glycosylation, one of which is used inefficiently (3, 23, 34). Lack of glycosylation at this site may affect the conformation of *Wnt*-1 proteins and lead to BiP binding.

The lumen of the ER is the site of a number of enzymes responsible for the posttranslational modification of secretory proteins, including signal peptidase, oligosaccharyl transferase and glucosidase, prolyl and lysyl hydroxylases, and protein disfulfide isomerase (8). It is possible that in the cell lines we have analyzed, one or more of these activities is not present in sufficient quantities to fully process *Wnt*-1 proteins and allow proper folding within the ER. For instance, 23 of the 370 primary amino acids of *Wnt*-1 are cysteines; attaining the correct disulfide-bonded state of such a cysteine-rich protein may be a difficult task. Protein disulfide isomerase catalyzes native disulfide bond formation during secretory protein biosynthesis (8) and is found in abundance in secretory cells, but it may be insufficiently represented in the cell lines we have analyzed. Alternatively, *Wnt*-1 proteins may require oligomerization via disulfide bonds in order to achieve a correct conformation. Disulfide-linked multimers of *Wnt*-1 have been detected (33); however, these multimers are thought to form as a consequence of overexpression of *Wnt*-1 proteins, and there is as yet no direct evidence that *Wnt*-1 multimers are the active forms of the protein.

Wnt-1 proteins may require association with other proteins in order to form secretion-competent or correctly folded proteins; however, extracellular *Wnt*-1 proteins have not been found in association with other proteins (2, 21, 35). The *Wnt*-1 gene is normally expressed in the developing nervous systems of mouse embryos and in spermatids of adult mouse testes (43, 51). Factors which either process or bind specifically to *Wnt*-1 proteins and allow the native conformation to be achieved may be expressed only in these tissues. Efforts to determine whether BiP is associated with endogenous *Wnt*-1 proteins in embryos and testes might help to resolve such questions.

ACKNOWLEDGMENTS

We are grateful to members of the *Wnt* group for discussions and advice. We also thank David Bole for the anti-BiP monoclonal antibody, Mario Chamorro for the generation of the anti-*Wnt*-1 rabbit serum C34, Helen Kwan for providing a *Wnt*-1 transgenic mouse mammary tumor, Neil Parkin for the pON *Wnt*-1 plasmid, and Greg Shackleford for the generation of the 7R and 7dT cell lines.

This work was supported by grants from the NIH (to H.E.V.). J.K. was a Fellow of The Jane Coffin Childs Memorial Fund for Medical Research and is now an American Cancer Society (California division) Senior Fellow, J.O.M. was an EMBO Long Term Fellow, and H.E.V. is an American Cancer Society Research Professor.

REFERENCES

1. **Bole, D. G., L. M. Hendershot, and J. F. Kearney.** 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting hybridomas. J. Cell Biol. **102**:1558–1566.
2. **Bradley, R. S., and A. M. C. Brown.** 1990. The proto-oncogene *int*-1 encodes a secreted protein associated with the extracellular matrix. EMBO J. **9**:1569–1575.
3. **Brown, A. M. C., J. Papkoff, Y. K. T. Fung, G. M. Shackleford, and H. E. Varmus.** 1987. Identification of protein products encoded by the proto-oncogene *int*-1. Mol. Cell. Biol. **7**:3971–3977.
4. **Brown, A. M. C., R. S. Wildin, T. J. Prendergast, and H. E. Varmus.** 1986. A retrovirus vector expressing the putative mammary oncogene *int*-1 causes partial transformation of a mammary epithelial cell line. Cell **46**:1001–1009.
5. **Dorner, A. J., D. G. Bole, and R. J. Kaufman.** 1987. The relationship of N-linked glycosylation and heavy chain binding protein association with the secretion of glycoproteins. J. Cell. Biol. **102**:2665–2674.
6. **Flynn, G. C., T. G. Chappell, and J. E. Rothman.** 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. Science **245**:385–390.
7. **Flynn, G. C., J. Pohl, M. T. Flocco, and J. E. Rothman.** 1991. Peptide-binding specificity of the molecular chaperone BiP. Nature (London) **353**:726–730.
8. **Freedman, R. B.** 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell **57**:1069–1072.
9. **Fung, Y.-K. T., G. M. Shackleford, A. M. C. Brown, G. S. Sanders, and H. E. Varmus.** 1985. Nucleotide sequence and expression in vitro of cDNA derived from mRNA of *int*-1, a provirally activated mouse mammary oncogene. Mol. Cell. Biol. **5**:3337–3344.
10. **Gavin, B. J., and A. P. McMahon (Roche Institute).** 1990. Personal communication.
11. **Gavin, B. J., J. A. McMahon, and A. P. McMahon.** 1990. Expression of multiple novel *Wnt*-1/*int*-1-related genes during fetal and adult mouse development. Genes Dev. **4**:2319–2332.
12. **Geballe, A. P., R. R. Spaete, and E. S. Mocarski.** 1986. A *cis*-acting element within the 5′ leader of a cytomegalovirus β transcript determines kinetic class. Cell **46**:865–872.
13. **Gething, M. J., K. McCammon, and J. Sambrook.** 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell **46**:939–950.
14. **Gething, M.-J., and J. Sambrook.** 1990. Transport and assembly processes in the endoplasmic reticulum. Semin. Cell. Biol. **1**:65–72.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
16. **Haas, I. G., and M. Wabl.** 1983. Immunoglobulin heavy chain binding protein. Nature (London) **306**:387–389.
17. **Hendershot, L., D. Bole, G. Kohler, and J. F. Kearney.** 1987. Assembly and secretion of heavy chains that do not associate posttranslationally with immunoglobulin heavy chain-binding protein. J. Cell. Biol. **104**:761–767.
18. **Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and C. S. Copeland.** 1989. Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). J. Cell Biol. **108**:2117–2126.
19. **Jue, S. F., R. S. Bradley, J. A. Rudnicki, H. E. Varmus, and A. M. C. Brown.** The mouse *Wnt*-1 gene can act via a paracrine mechanism in transformation of mammary epithelial cells. Mol. Cell. Biol. **12**:321–328.
20. **Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly.** 1988. Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. Nature (London) **333**:90–93.
21. **Kitajewski, J., and H. E. Varmus.** Unpublished data.
22. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High-level expression of a chloramphenicol acetyltransferase gene by DEAE-dextran-mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
23. **Mason, J. O., J. Kitajewski, and H. E. Varmus.** Unpublished data.
24. **McMahon, A. P., and A. Bradley.** 1990. The *Wnt*-1 (*int*-1) proto-oncogene is required for development of a large region of the mouse brain. Cell **62**:1073–1085.
25. **McMahon, A. P., and R. T. Moon.** 1989. *Int*-1—a proto-oncogene involved in cell signalling. Development **107**(Suppl.): 161–167.
26. **McMahon, J. A., and A. P. McMahon.** 1989. Nucleotide sequence, chromosomal localization and developmental expression of the mouse *int*-1-related gene. Development **107**:643–650.
27. **Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. C. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese

quail. Cell 11:95–103.
28. **Munro, S., and H. R. B. Pelham.** 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46:291–300.
29. **Ng, D. T. W., S. W. Hiebert, and R. A. Lamb.** 1990. Different roles of individual N-linked oligosaccharide chains in folding, assembly, and transport of the simian virus 5 hemagglutinin-neuraminidase. Mol. Cell. Biol. 10:1989–2001.
30. **Nguyen, T. H., D. T. S. Law, and D. B. Williams.** 1991. Binding protein BiP is required for translocation of secretory proteins into the endoplasmic reticulum in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 88:1565–1569.
31. **Nusse, R., A. van Ooyen, D. Cox, Y. K. T. Fung, and H. E. Varmus.** 1984. Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature (London) 307:131–136.
32. **Nusse, R., and H. E. Varmus.** 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31:99–109.
33. **Papkoff, J.** 1989. Inducible overexpression and secretion of *int*-1 protein. Mol. Cell. Biol. 9:3377–3384.
34. **Papkoff, J., A. M. C. Brown, and H. E. Varmus.** 1987. The *int*-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway. Mol. Cell. Biol. 7:3978–3984.
35. **Papkoff, J., and B. Schryver.** 1990. Secreted *int*-1 protein is associated with the cell surface. Mol. Cell. Biol. 10:2723–2730.
36. **Parkin, N., J. Kitajewski, and H. E. Varmus.** Unpublished data.
37. **Pelham, H. R. B.** 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell 46:959–961.
38. **Rijsewijk, F., M. Schuermann, E. Wagenaar, P. Parren, D. Weigel, and R. Nusse.** 1987. The *Drosophila* homolog of the mouse mammary oncogene *int*-1 is identical to the segment polarity gene *wingless*. Cell 50:649–657.
39. **Rijsewijk, F., L. van Deemter, E. Wagenaar, A. Sonnenberg, and R. Nusse.** 1987. Transfection of the *int*-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J. 6:127–131.
40. **Roelink, H., and R. Nusse.** 1991. Expression of two members of the *Wnt* family during mouse development—restricted temporal and spatial patterns in the developing neural tube. Genes Dev. 5:381–388.
41. **Roelink, H., E. Wagenaar, S. L. Da Silva, and R. Nusse.** 1990. *Wnt*-3, a gene activated by proviral insertion in mouse mammary tumors, is homologous to *int*-1/*Wnt*-1 and is normally expressed in mouse embryos and adult brain. Proc. Natl. Acad. Sci. USA 87:4519–4523.
42. **Rothman, J. E.** 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59:591–601.
43. **Shackleford, G. M., and H. E. Varmus.** 1987. Expression of the proto-oncogene *int*-1 is restricted to postmeiotic male germ cells and the neural tube of mid-gestational embryos. Cell 50:89–95.
44. **Sitia, R., M. Neuberger, C. Alberini, P. Bet, A. Fra, C. Valetti, G. Williams, and C. Milstein.** 1990. Developmental regulation of IgM secretion: the role of the carboxy-terminal cysteine. Cell 60:781–790.
45. **St.-Arnaud, R., J. Craig, M. W. McBurney, and J. Papkoff.** 1989. The *int*-1 proto-oncogene is transcriptionally activated during neuroectodermal differentiation of P19 mouse embryonal carcinoma cells. Oncogene 4:1077–1080.
46. **Thomas, K. R., and M. R. Capecchi.** 1990. Targeted disruption of the murine *int*-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. Nature (London) 346:847–850.
47. **Tsukamoto, A. S., R. Grosschedl, R. C. Guzman, T. Parslow, and H. E. Varmus.** 1988. Expression of the *int*-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 55:619–625.
48. **Vaidya, A. B., E. Y. Lasfargues, J. B. Sheffield, and W. G. Coutinho.** 1978. Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. Virology 90:12–22.
49. **van Ooyen, A., and R. Nusse.** 1984. Structure and nucleotide sequence of the putative mammary oncogene *int*-1; proviral insertions leave the protein-encoding domain intact. Cell 39: 233–240.
50. **Vogel, J. P., L. M. Misra, and M. D. Rose.** 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. J. Cell Biol. 110:1885–1895.
51. **Wilkinson, D. G., J. A. Bailes, and A. P. McMahon.** 1987. Expression of the proto-oncogene *int*-1 is restricted to specific neural cells in the developing mouse embryo. Cell 50:79–88.